Analysis of Photochemical Compounds in *Moringa oleifera* Leaves

with Capillary Zone Electrophoresis

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List of Abbreviations

BAW	n-butanol/acetic acid/water solvent system
CE	Capillary Electrophoresis
CZE	Capillary Zone Electrophoresis
EtOH	Ethanol
HOAc	Acetic Acid
I	Isoquercetin
MO	Moringa oleífera
Q	Quercetin
R	Refluxed
R	Rutin
S	Shaken
UV	Ultra-violet

Abstract

Moringa oleifera is a tropical plant that has been traditionally used as a dietary supplement and medicinal plant due to its rich composition of protein, minerals, vitamins, and phytochemicals. All the parts of the plant can be used, but previous research has suggested that consuming the leaves of *Moringa oleifera* has positive health benefits due to the bioactive compounds present, therefore, the identification of bioactive compounds, either acting alone or in combination with other compounds is needed. A literature review on previously identified in *Moringa oleifera* leaves resulted in 168 phytochemical compounds identified which have anti-inflammatory, antimicrobial, antioxidant, anti-cancer, hypoglycemic, and negative bioactivities. These compounds can be further classified as phenolics and flavonoids. This study focuses on the phytochemicals that interact with light in the UV and visible regions, or photochemicals. Of interest are compounds that absorb in the phenolic (235-335 nm) and flavonoid regions (225-430 nm). Ethanolic and aqueous extracts of *Moringa oleifera* leaves have been used to examine photochemicals with absorbance signals to identify the specific compounds present and exploit them as standards. Paper chromatography results suggest the presence of flavonoids in *Moringa oleifera* leaves. The absorbance and total phenolic content results suggest that the optimal extraction method was shaking in 80% ethanol with a total phenolic content of 8.6 ± 0.1 mM. Capillary zone electrophoresis with absorbance detection at 340 nm was used to analyze the flavonoid compounds in the *Moringa oleifera* leaf extract solutions and isoquercetin and rutin were suggested to be present, while p-coumaric acid was most likely not present. The concentration of isoquercetin in Moringa oleifera leaves was calculated to be 459 ± 78 μM. Using capillary zone electrophoresis will assist with identification of bioactive compounds that promote positive health effects.

Introduction

Background

Moringa oleifera is one of the many medicinal herbs that has been used for centuries to treat ailments. Belonging to the Moringaceae family, Moringa has thirteen species, but the most common is *Moringa oleifera*.[1] *Moringa oleifera* grows under many conditions, but it prefers a dry to moist tropical climate with temperatures between 18°C and 28°C, 760-2500 mm of precipitation annually, heavy clay soil, and at an altitude below 2000 m.[1] *Moringa oleifera* is indigenous to the foothills of the Himalayas, but it has spread across the tropics after 1990 when some researchers began studying the plant and reported the many benefits of consuming the plant.[1-2]

All the parts of the tree are considered edible and have been traditionally used to treat many ailments and conditions, such as malaria, arthritis, skin diseases, genito-urinary ailments, hypertension, diabetes, and digestion aid.[1] *Moringa oleifera* is not only known for its medicinal properties, but it is also considered a nutritional supplement due to its high levels of protein and fat.[1] The leaves are consumed as a part of the diet or as treatments for acute and chronic conditions.

Phytochemical categorization

One of the characteristics of *Moringa oleifera* is its high abundance of phytochemicals. Phytochemicals are biologically active organic compounds that are synthesized and accumulated in the plant cell.[1, 3] Phytochemicals have been found to provide positive health benefits, including anti-oxidant, anti-inflammatory, anticancer, and antimicrobial properties.[4] These properties of phytochemicals could be the source of the medicinal qualities of *Moringa oleifera*.

There are many types of phytochemicals that can be further classified by their chemical structure as diagramed in Figure 1. This study will focus on phenolic compounds, because they are primarily responsible for the antioxidant activity of medicinal plants.[5] Phenols and phenolic acids are rarely unbound within the cell, and are usually stored as glucosides.[3] One example of free and glycosylated phenolic acids are quercetin and isoquercetin, where isoquercetin is a quercetin molecule that is bound to glucose (Figure 2). Flavonoids is the largest category of phenolic compounds. Flavonoids are derived from flavone and have been found to be effective antioxidants.[3, 6] Anthocyanidins, chalcones, and aurones are colored complexes, while the other compounds do not absorb light within the visible spectrum.[3]

Previous research has identified 168 compounds in *Moringa oleifera*, while 66 of those compounds were considered bioactive.[1, 7-8] The bioactivity of the compounds was defined by effects at a cellular or systematic level (Table 1). In total, 15 compounds had antioxidant properties, 14 had anti-cancer properties, 9 had antimicrobial properties, 8 had anti-inflammatory properties, 7 had cardioprotective properties, 3 had hypoglycemic properties, and 7 had negative properties (Figure 3). Some of the compounds had multiple bioactivities. Most of the compounds with bioactivity were classified as phenolic, but they do not account for all the bioactive compounds. Flavonoids and phenylpropanoids account for most of the phenolic compounds. Flavonoids had similar bioactivities as the total compounds, except that they did not account for any cardioprotective activity (Figure 4). Even though many of the compounds present in *Moringa oleifera* are considered to have positive health effects, there were some compounds that were considered to have negative bioactivities by being harmful, toxic, or causing irritation. The negative effects were commonly paired with a positive bioactivity.

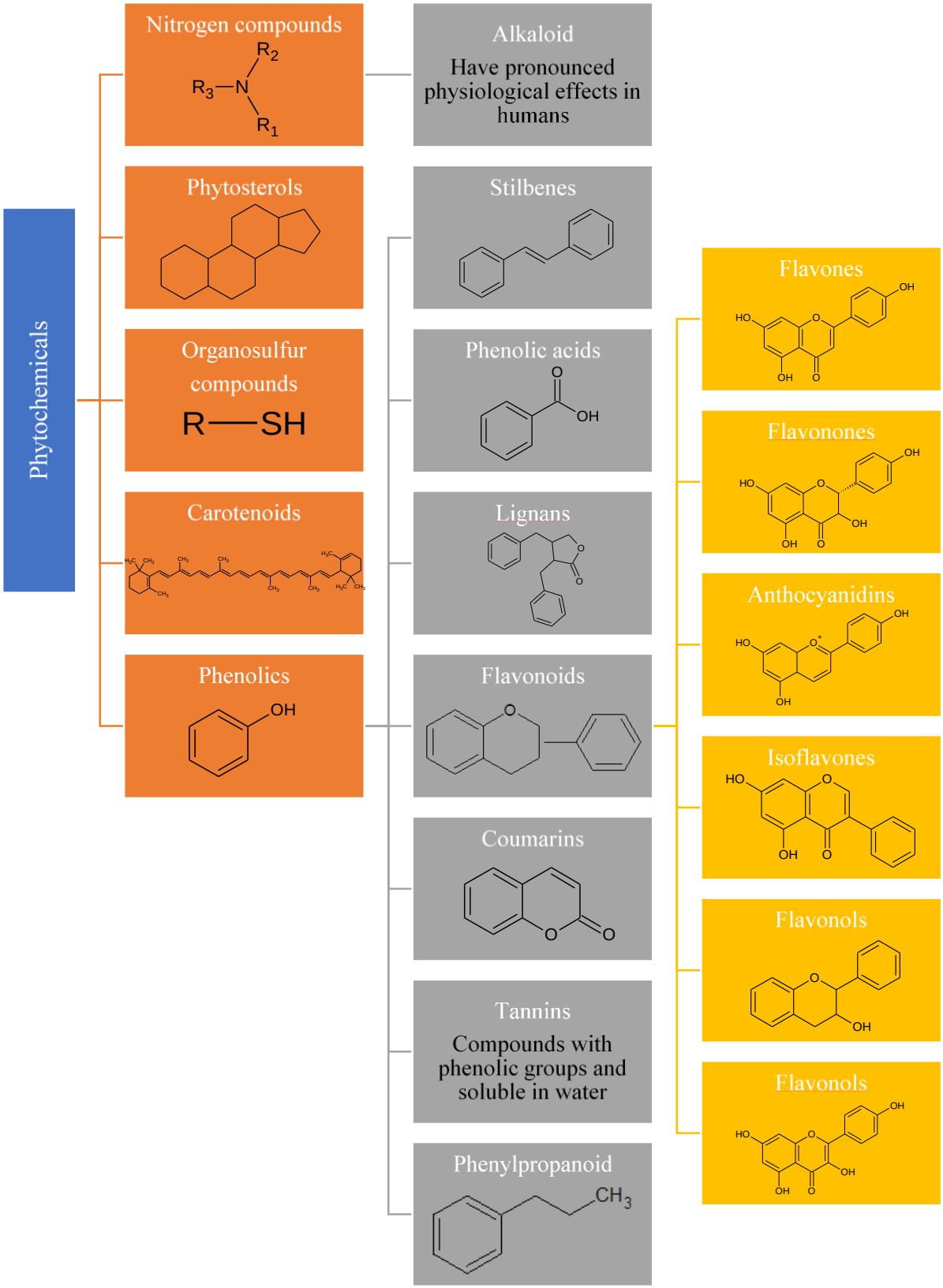

Figure 1: Phytochemical categorization based on structure adopted from Harborne and Higdon et al.[3, 9-10]

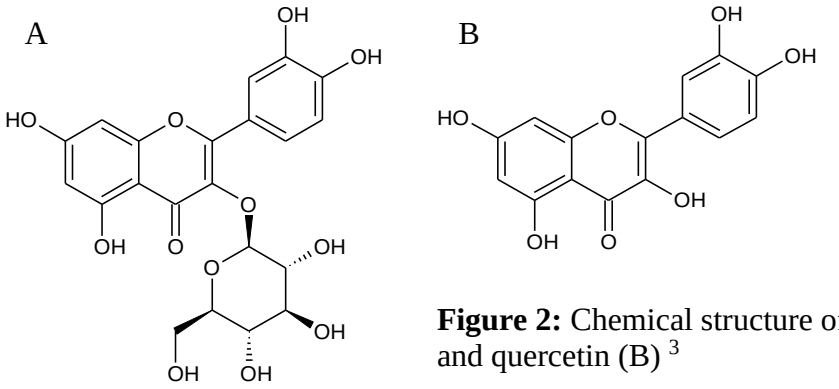

Figure 2: Chemical structure of isoquercetin (A) and quercetin (B) [3]

Table 1: Definition of bioactivity terms used in Figures 3 and 4.

Category	**Definition**
Negative	Causes eye, respiratory, or skin irritation, toxic to aquatic life, toxic or harmful if swallowed, suspected of causing cancer, suspected of infertility activity or harmful to fetus
Hypoglycemic	Hypoglycemic activity, antihyperglycemic activity, increased glycogen synthesis, induction of glucose reuptake, insulin mimicking activity
Anti-cancer	Inhibition of tumor promoter, inhibition of cell proliferation, induction of apoptosis, anti-tumor activity, tumor inhibitor, inhibitor of myeloid cell leukemia sequence 1, induces differentiation and apoptosis of tumor cells, reduced risk of stomach cancer, antiproliferative, agonist against p53 pathway, tumor cell line growth inhibition
Antioxidant	Antioxidant activity
Antimicrobial	Antiviral against HIV1, hepatitis B. Inhibition of *Saccharomyces cerevisiae* alcohol dehydrogenase, antibiotic, prevents spread of infection, disruption of bacteria and viruses by attaching to cell wall, neutralizes toxins from bacteria
Anti-inflammatory	Anti-inflammatory activity
Cardioprotective	Hypotensive activity, antihypertensive agent, inhibition of LDL oxidation, hypocholesterolemic activity

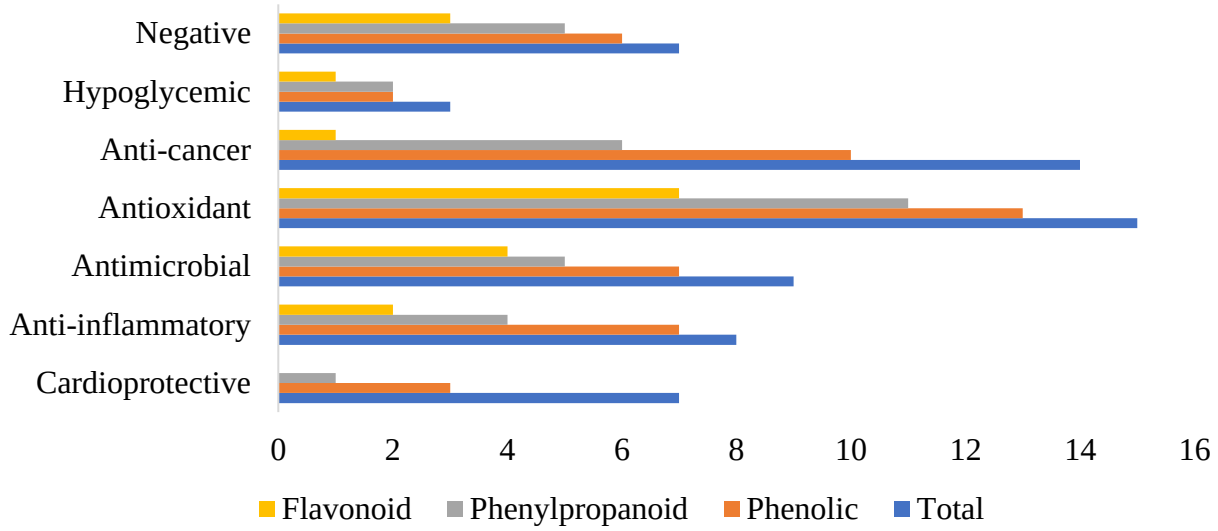

Figure 3: Bioactivity of previously identified phytochemicals by category in *Moringa oleifera*

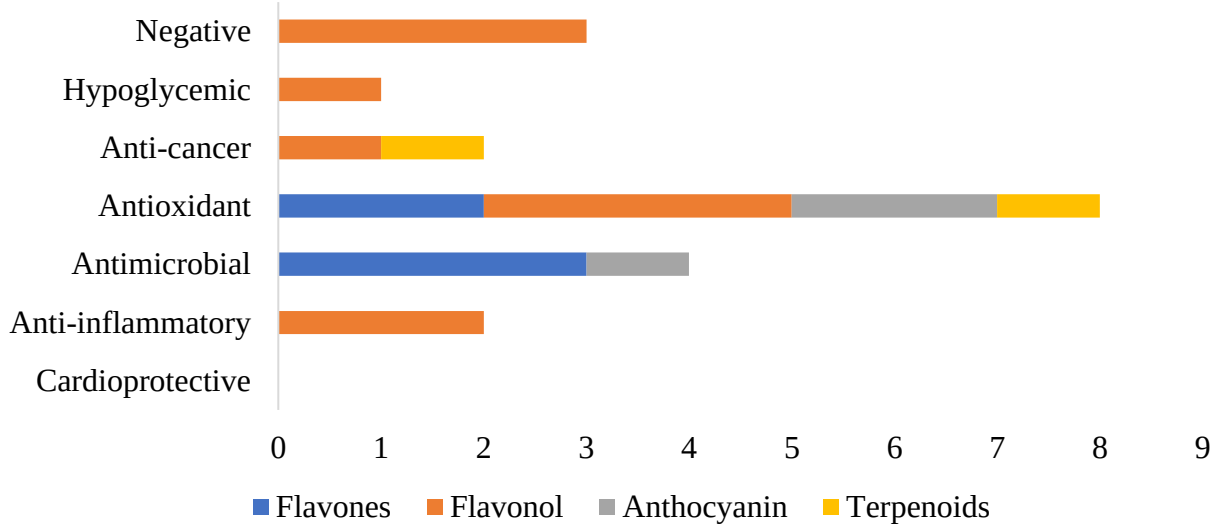

Figure 4: Bioactivity of previously identified flavonoids by category in *Moringa oleifera*

Extraction

The extraction process is crucial to the phytochemical analysis of the *Moringa oleifera* plant. Polar solvents, such as methanol, ethanol, acetone, and ethyl acetate, are the most commonly used to extract the phytochemicals from the plant material. Sultana et al. compared absolute methanol, 80% aqueous methanol, absolute ethanol, and 80% aqueous ethanol as

solvents.[11] They also evaluated the influence of the technique of extraction, refluxing or shaking, on the resulting extract. When evaluating the total extract yield of *Moringa oleifera* leaves, they found that refluxing the extract in methanol resulted in the highest yield.[11] On the other hand, the total phenolic and flavonoid content was highest when the *Moringa oleifera* leaves were extracted in aqueous methanol and shaken. The higher phenolic and flavonoid content in the extracts that were shaken compared to the refluxed extracts was associated with compound degradation by the heat during the extraction.

Maqsood et al. studied the effect of the polarity of the solvent on the presence of phytochemicals in the extract and on their antifungal activity.[12] The same plant material was extracted in solvents of increasing polarity, and each solvent was studied for the presence of phytochemicals. The order of solvents was n-hexane, chloroform, acetone, ethyl acetate, ethanol, methanol, and water. Flavonoids, saponins, alkaloids, phenolics, and tannins were mainly present in the ethanolic and methanolic extracts. The total phytochemical quantities by category in *Moringa oleifera* were determined as flavonoids (21.76±0.68%), tannins (14.3±0.26%), saponins (12.56±0.51%), and alkaloids (2.4±0.85%).

Separation and detection techniques

The analytical methods in this study use light to detect the compounds of interest. Therefore, this study will focus on photochemicals, or phytochemicals that interact with light. Absorbance spectroscopy on the whole extract was performed to analyze the general composition of the *Moringa oleifera* leaves. Phenolic compounds are expected to absorb at 235-335 nm, which overlaps with the expected absorbance of flavonoids at 225-430 nm.[3] Both

phenolics and flavonoids absorb within the ultraviolet (UV) region which is expected due to their aromaticity.

Paper chromatography

Paper chromatography is a simple, convenient, inexpensive, and reproducible technique to analyze compounds in a mixture.[3] The properties of the compound and its interaction with the solvent determine the distance traveled by the compound, and the retention factor can be used to identify a compound. The retention factor (R_f) is calculated by dividing the distance traveled by the compound by the total distance traveled by the solvent. Sankhalkar and Vernekar determined the presence of biflavonyls (kayaflavone) in *Moringa oleifera* leaves using paper chromatography with n-butanol/acetic acid/water (4:1:5, BAW) as the solvent system.[4] This study used paper chromatography to analyze the major photochemicals in *Moringa oleifera* leaves using various solvent systems.

Capillary electrophoresis

Capillary electrophoresis (CE) is a low cost analytical tool that uses an electrical current to separate compounds.[13] One type of CE, capillary zone electrophoresis (CZE), separates the compounds according to their differences in size and charge as they travel through the capillary by the electroosmotic flow (Figure 5).[14] The electroosmotic flow is the bulk movement of the buffer or background electrolyte as the electrical current is applied to the capillary, this electroosmotic flow aids in the movement of the compounds through the capillary independent of charge.[15] Since the compounds are moving towards the negative electrode, the positive compounds will elute first, and the negative compounds elute last following the pattern shown in

Figure 6. The mobility of a compound is therefore dependent on the electroosmotic flow and the electrophoretic mobility, which is dependent on the charge and size of the compound. Effective separation can be achieved between phenolic compounds using CZE, therefore it is an adequate technique to study the phytochemical composition of plant extracts.[16-17] Previous studies have effectively used CZE to study the composition of *Aloe vera*, hawthorn, mulberry leaves, wheat, *brassica oleracea, R. erythrocladus*, and *M. nigra*.[5, 17-21] To date, there has not been a phytochemical analysis of *Moringa oleifera* leaves using capillary zone electrophoresis. Since absorbance detection is used instead of mass spectrometry detection, standard compounds can be used as a reference to identify the peaks present in the electropherograms of the sample. Some of the advantages to using capillary zone electrophoresis is that only a small amount of sample is required to achieve separation and it can be completed in a relatively short amount of time. One of the disadvantages of using capillary zone electrophoresis is that compounds with similar charges and sizes could be difficult to separate.

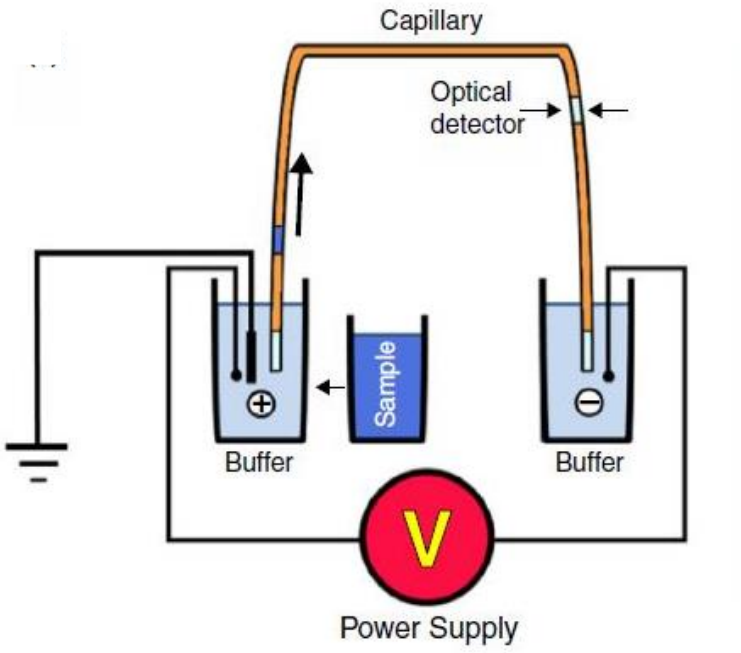

Figure 5: Basic setup of capillary electrophoresis taken from Holtkamp and Hartinger [22]

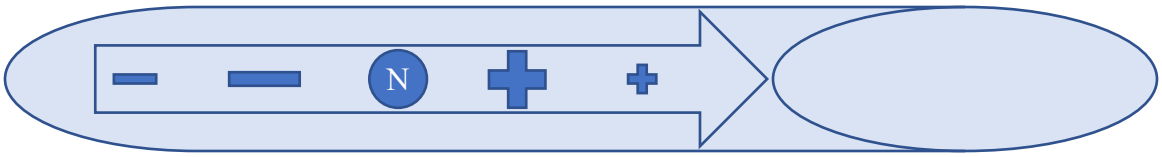

Figure 6: Separation of compounds by size and charge in capillary zone electrophoresis (-, negatively charged species; N, neutral species; +, positively charged species)

Several researchers have studied the phenolic content in other plant material with CZE and optimized the conditions for analysis.[5, 17-21] All the researchers used a 50 µm capillary for the CZE analysis. The temperature the separation occurred at 25°C or 30°C for the study of phenolics and flavonoids. The background electrolytes used by previous studies of phenolic compounds were 15 mM and 40 mM borate buffer, and 50 mM borate buffer in 20% methanol (v/v), in the study of flavonoids 40 mM and 50 mM borate buffer, and 20 mM borate buffer with 10% acetonitrile were used as background electrolytes. The pH of the buffers ranged from 8.2 to 9.3 to study phenolics and flavonoids. The optimized injection pressures were 50 mbar for 5 seconds, 0.5 psi over 8 seconds, and 300 mbar. The separation voltage ranged from 20 kV to 30 kV. Phenolics were detected at 200 nm, 300 nm, and 320 nm while flavonoids were detected at 210 nm, 254 nm, and 280 nm.

The purpose of this experiment is to use paper chromatography and capillary zone electrophoresis using UV absorbance detection to analyze the photochemical content in *Moringa oleifera* leaves. A greater understanding of the photochemical content of *Moringa oleifera* leaves will provide a greater insight into the sources of the many bioactivities of *Moringa oleifera*. The results from this experiment will also help the study of the environmental and genetic factors that affect the photochemical composition of *Moringa oleifera*.

Experimental Methods

Chemicals and materials

Borate buffer was prepared with sodium tetraborate (CAS # 1330-43-4) and then made into 10 mM concentration. Quercetin (CAS # 117-39-5) and rutin (CAS # 153-18-4) were used as standards and the solutions were prepared by dissolving them in absolute ethanol. Acetic acid, concentrated hydrochloric acid, n-butanol, and sodium hydroxide (1 M) were obtained from the Appalachian State University Chemistry Department. Folin-Ciocalteu's Phenol Reagent (Sigma F9252-100) and gallic acid (CAS # 149-91-7) were used to measure the total phenolic content in *Moringa oleifera* leaves. Deionized (DI) water was used throughout the experiment. The *Moringa oleifera* leaves were dried and ground into a fine powder. The plant material was stored in a glass jar and refrigerated until use. Class A glassware was used throughout the experiment, and it was washed with warm soapy water and rinsed with DI water. Eppendorf micropipettes were also used to perform volume measurements.

Extraction

Moringa oleifera powder (1 g) was extracted with 25.00 mL of solvent and then refluxed (45 min) or shaken (45 min, 4 hrs, or 6 hrs) as diagramed in Figure 7. The extracted samples were vacuum filtered and vacuum dried to evaporate all the solvent. The extract was resuspended in 9 mL of DI water and mixed with 100 mM borate buffer, then diluted to 10 mL. The resuspended extract was filtered with 0.22 µm filter to filter any material that could clog the capillary and stored in the freezer until further analysis.

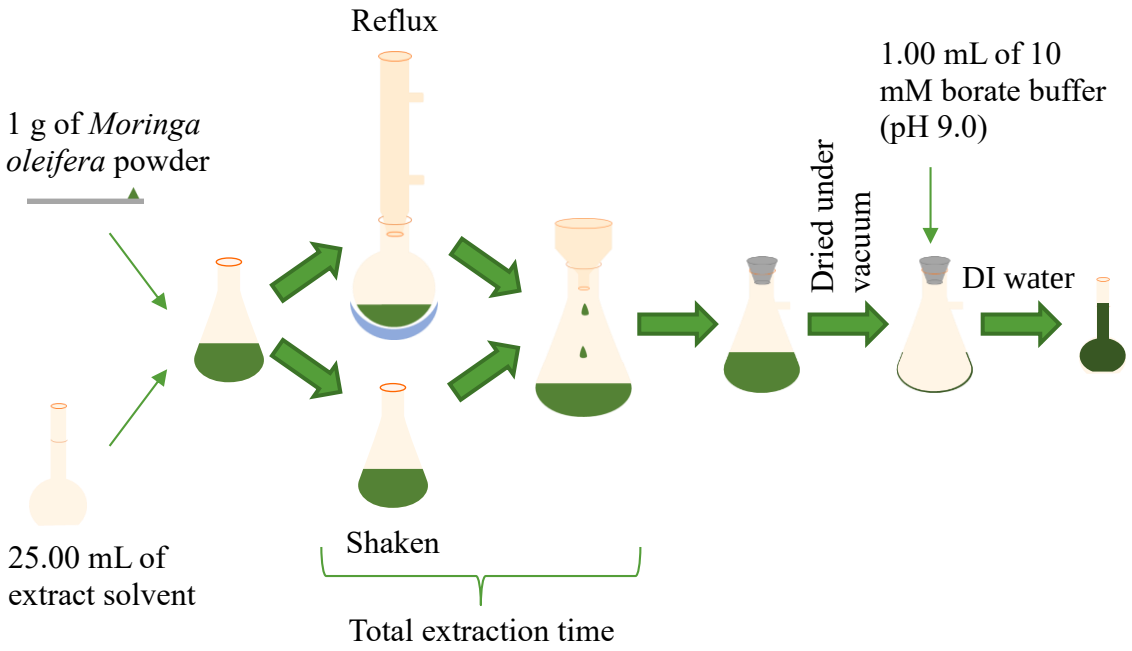

Figure 7: Extraction method for *Moringa oleifera* leaves

Absorbance

The UV Vis absorbance (190 nm-840 nm) of various samples at a 1:150 (v/v) dilution were measured on a Thermo Fisher Nanodrop spectrophotometer.

Paper chromatography

The photochemicals in whole ethanolic *Moringa oleifera* extract were separated using ascending paper chromatography on #1 Whatman paper. The solvent systems used were Forestal (acetic acid, conc. HCl, water 30:3:10, v/v), 50% acetic acid (v/v), BAW (n-butanol, acetic acid, water 4:1:5, v/v) and water. The separated compounds were compared to quercetin and rutin standards in ethanol and were viewed under 365 nm light using the UVP ChemiDoc-It Imager.

Total phenolic content

The total phenolic content of the *Moringa oleifera* samples was determined using the Folin-Ciocalteu's phenol reagent and used to calculate the total phenolic content.[23] Samples were prepared on a 96 well plate and the absorbance was measured at 595 nm. Spectrophometric readings were completed on a Tecan Infinite F500 plate reader and Magellan software was used for collection. A stock solution of gallic acid was prepared by dissolving gallic acid in water with 1 mL of 100 mM borate buffer and diluted to 10 mL. Standard solutions of gallic acid were prepared with 0 μL, 1 μL, 3 μL, 5 μL, 8 μL, 11 μL, and 15 μL of gallic acid stock solution and diluted to 15 μL with 10 mM borate buffer. The experimental samples were prepared with 15 μL of diluted *Moringa oleifera* sample solutions, then all the solutions were diluted with 40 μL of DI water and mixed with 15 μL of Folin-Ciocalteu phenol reagent. After 5 minutes, the solutions were mixed with 65 μL of sodium carbonate (8%) and diluted to 200 μL with DI water. The *Moringa oleifera* samples were blanked with a *Moringa oleifera* solution that did not contain the Folin-Ciocalteu phenol reagent. Total phenolic content in the samples was calculated as a gallic acid equivalent based on a standard curve with the equation $y = (0.00652 \pm 0.00007)x$ and an R squared value of 0.9995. Rutin and quercetin were used as internal standards for quality control. All measurements were completed in triplicate.

Capillary electrophoresis

Capillary electrophoresis was completed using a 50 μm x 59.8 cm capillary at 25°C on Beckman Coulter P/ACE MDQ Capillary Electrophoresis System. The capillary was rinsed with 1 M NaOH, DI water, and 10 mM borate buffer (pH = 9.0) at 40 psi for 3 min each before every sample injection. The sample was injected at 1.0 psi for 5.0 sec and separated with running

borate buffer at 20.0 kV for 12.00 min with detection at 340 nm. Acetone was used as the neutral marker and added to the solutions in 0.14 M concentration.

Isoquercetin standard solution preparation

Five standard solutions were prepared with 5 µL, 50 µL, 125 µL, and 250 µL of 200 µM isoquercetin solution then mixed with 100 µL of acetone, and the equivalent volume of 100 mM borate buffer for all solutions to have a final borate concentration of 10 mM, then water was used to dilute all samples to 1 mL. The solutions had final isoquercetin concentrations of 0 µM, 1.0 µM, 10.0 µM, 25.0 µM, and 50.0 µM. An internal standard was prepared by mixing 500 µL of *Moringa oleifera* sample in 10 mM borate buffer with 100 µL of acetone, 100 µL of isoquercetin in 10 mM buffer, 40 µL of 100 mM borate buffer and diluted to 1 mL with DI water.

Data Analysis

The data in the experiment was analyzed on Microsoft Excel. Standard deviation was used as the uncertainty in the paper chromatography retention factors. The uncertainty in the isoquercetin concentration was calculated using Equation 1.

$$u_x = \frac{s_y}{|m|}\sqrt{\frac{1}{n} + \frac{\bar{y}^2}{m^2 \sum(x_i - \bar{x})^2}} \qquad \textit{Equation 1}$$

Results and Discussion

Paper Chromatography

The ethanolic extracts of *Moringa oleifera* leaves were studied using paper chromatography and were compared to quercetin and rutin standards, because they represented a free flavonoid and a glycosylated flavonoid (Figure 8). The retention factor (R_f) of rutin using water as the solvent system and quercetin using the Forestal solvent system agreed with the literature values within error.[3, 24] For the other retention factors, the other literature values were unknown or did not agree with the literature value within error (Table 2). One trial of the *Moringa oleifera* leaves using water as the solvents system, had a compound ($R_f = 0.10$) which could suggest the presence of astragalin ($R_f = 0.13$), daidzein ($R_f = 0.08$), or isoquercetin ($R_f = 0.08$). Separation of compounds in *Moringa oleifera* leaf extract with 50% acetic acid resulted in three visible compounds (mean ± sd), $R_f = 0 \pm 0.01$, $R_f = 0.70 \pm 0.02$, and $R_f = 0.94 \pm 0.04$, these values do not match any literature values. Using the BAW (n-butanol, acetic acid, water, 4:1:5 v/v) solvent system, the S2 fraction of the *Moringa oleifera* extract was also analyzed, and no flavonoids were visible while two compounds were visible for the whole extract at $R_f = 0.69 \pm 0.05$ and $R_f = 1.00 \pm 0.01$ which could suggest the presence of astragalin ($R_f = 0.70$), isorhamnetin ($R_f = 0.74$), and genistein ($R_f = 0.94$). The results suggest the presence of photochemicals in *Moringa oleifera* leaves.

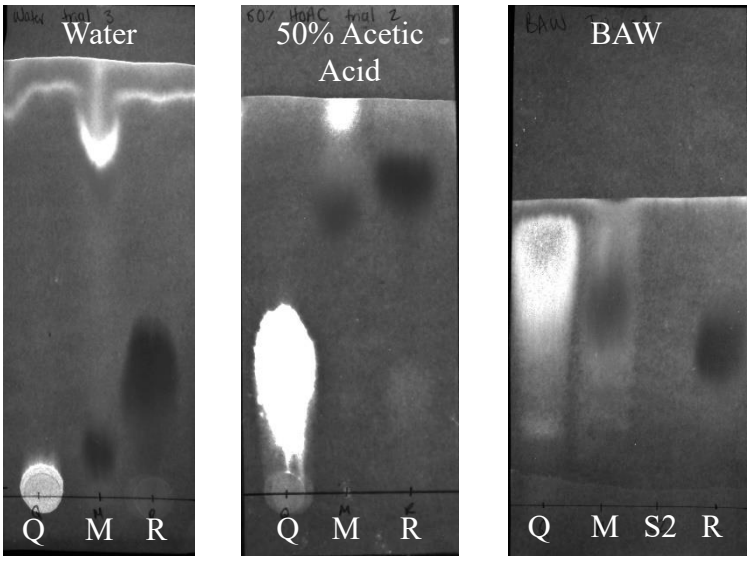

Figure 8: Paper chromatograph of quercetin (Q), *Moringa oleifera* leaves (M), and rutin (R) in water, 50% acetic acid, and BAW viewed under 365 nm light

Table 2: Average retention factors of quercetin, rutin, and compounds in *Moringa oleifera* extracts in water, 50% acetic acid, BAW, and forestal solvent systems

Solvent system	Quercetin	Rutin	Moringa Extract
Water	0.00 ± 0.01 (0.19)	0.29 ± 9 (0.23)	0.10* Smear
50% acetic acid	0.32 ± 0.03	0.80 ± 0.04	0.00 ± 0.01 0.70 ± 0.02 0.94 ± 0.04
BAW	0.84 ± 0.02 (0.64)	0.54 ± 0.02 (0.45)	0.69 ± 0.05 1.00 ± 0.01
Forestal	0.46 ± 0.04 (0.41)	0.81* Undetermined	0.76* 0.87* Undetermined

Mean ± standard deviation
*Only measured in one sample
(literature values)[3, 24]

Extraction Method Optimization

Absorbance

Most of the absorbance is occurring in the UV region, which suggests the presence of phenolic and flavonoid compounds in *Moringa Oleifera* leaves. The samples had maximum absorbance at 270 nm and 360 nm (Figures 9 and 10). Higher absorbance intensity implies higher concentration of photochemicals. Some of the samples had a peak at 675 nm which

correlated with a peak at 462 nm, which suggests the presence of chlorophyll in those samples.[3] There was a significant difference in the absorbance in the whole *Moringa oleifera* leaf extracts between the samples extracted in 80% ethanol and 100% ethanol by refluxing, with the solution extracted in 100% ethanol having a lower absorbance (Figure 9). There is also a significant decrease in the maximum absorbance at 360 nm in the sample of *Moringa oleifera* leaves extracted in 100% ethanol, which suggests a lower concentration of flavonoids in the sample. Even though acidified ethanol was suggested as a more effective extraction solvent, there was not a significant difference in the absorbance between the acidified and non-acidified extracts, except that the acidified extract had a decrease in the absorption at 462 nm and 675 nm, suggesting that the acidified extract contained a lower concentration of chlorophyll which suggests that the acid decreases the solubility of chlorophyll in the solution.[3] There was no significant difference in the absorbance of the samples extracted in 80% ethanol and shaken for 45 minutes, 4 hours, and 6 hours, suggesting that the total extraction time did not greatly impact the amount of photochemicals extracted (Figure 10). The sample of *Moringa oleifera* leaves extracted in 100% ethanol by shaking had a higher absorbance than the sample extracted in 80% ethanol by shaking. Overall, the sample with the most absorbance was the one extracted in 100% ethanol and shaken for 6 hours. The wavelengths of maximum absorbance were compared to previously identified compounds in *Moringa oleifera* to choose standards for quantitative methods.

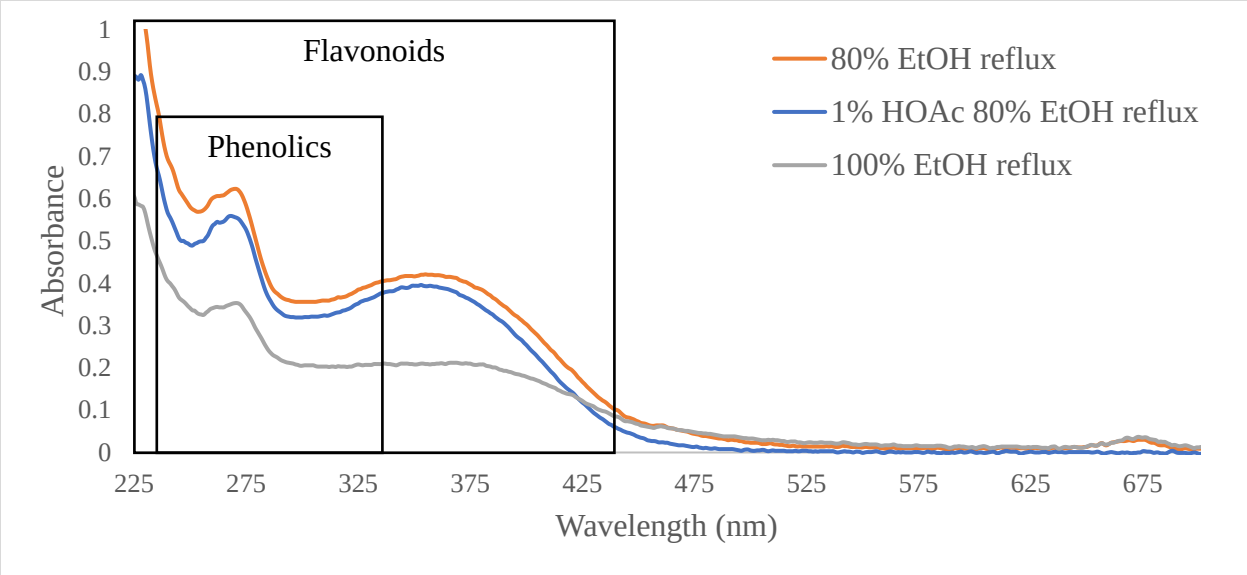

Figure 9: Absorbance spectra of whole *Moringa oleifera* leaves extracted in 80% ethanol, acidified 80% ethanol, and 100% ethanol by refluxing.

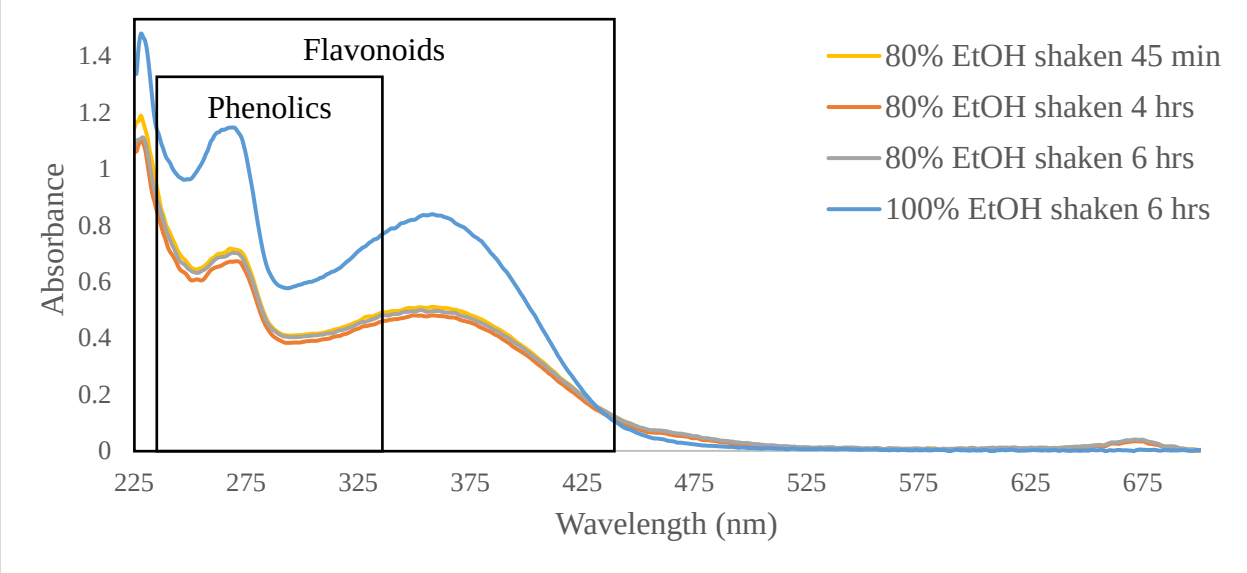

Figure 10: Absorbance spectra of whole *Moringa oleifera* leaves extracted in 80% ethanol and 100% ethanol by shaking.

Total phenolic content

The total phenolic content of the *Moringa oleifera* samples were calculated as a gallic acid equivalent using the Folin-Ciocalteu phenol reagent. Rutin (262 µM) and quercetin (331 µM) samples were used as quality control. The calculated phenolic concentration for rutin was

445 ± 13 μM and 741 ± 13 μM for quercetin. The difference between the actual and calculated concentration for rutin and quercetin could be accounted for by the fact that the Folin-Ciocalteu phenol reagent reacts with the compounds and gets oxidized, which results in the change in absorbance. The polyphenolic nature of rutin and quercetin results in greater antioxidant activity in the compounds, which could lead to an overestimation of the concentration of rutin and quercetin in the samples (Figure 11). These results suggest that the calculated total phenolic are overestimated.[25]

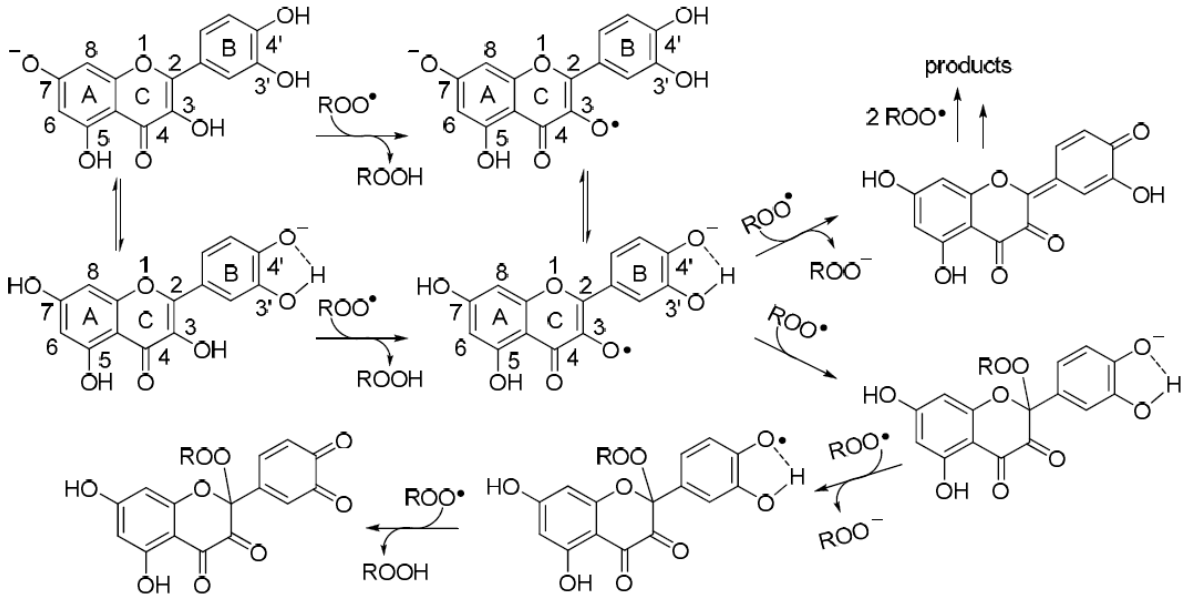

Figure 11: Proposed mechanism of quercetin antioxidant activity in an aqueous solution [26]

The *Moringa oleifera* samples extracted in 100% ethanol had a total phenolic concentration of 2.3 ± 0.1 mM for the refluxed and shaken extract (Figure 12). The refluxed extracts in 80% ethanol had a total phenolic concentration of 6.8 ± 0.1 mM for the sample extracted in February and 6.9 ± 0.1 mM for the sample extracted in May. These results agree within error, which suggests that the extraction processes are reproducible. The *Moringa oleifera* sample extracted in acidified (1% acetic acid) 80% ethanol by reflux had a total phenolic content

of 7.2 ± 0.1 mM, which does not agree within error with the sample extracted in non-acidified 80% ethanol by reflux, which suggests that the acidified extract could aid in the effectiveness of the solvent to extract the compounds from the sample. The extracts in 80% ethanol by shaking had a total phenolic content of 8.1 ± 0.1 mM (Apr), 7.2 ± 0.1 mM (May), 7.0 ± 0.1 mM (Jun 1), and 8.6 ± 0.1 mM (Jun 2). The samples Jun 1 and Jun 2 were completed on the same day; the difference in total phenolic content could be due to slight product loss for Jun 1 in the extraction process. The sample extracted in 1% hydrochloric acid 80% ethanol by shaking had a total phenolic content of 4.99 ± 0.1 mM, which does not agree within error with the non-acidified extract which could suggest degradation of products in the sample. Finally, the *Moringa oleifera* sample extracted in DI water by shaking had a total phenolic content of 5.7 ± 0.1 mM, which did not agree within error with the sample extracted in 100% ethanol. The total phenolic content of the water extract did not agree within error of the extracts in 80% ethanol by shaking. The results suggest that was is a better extraction solvent than 100% ethanol, but 80% ethanol was a more effective extraction solvent than water.

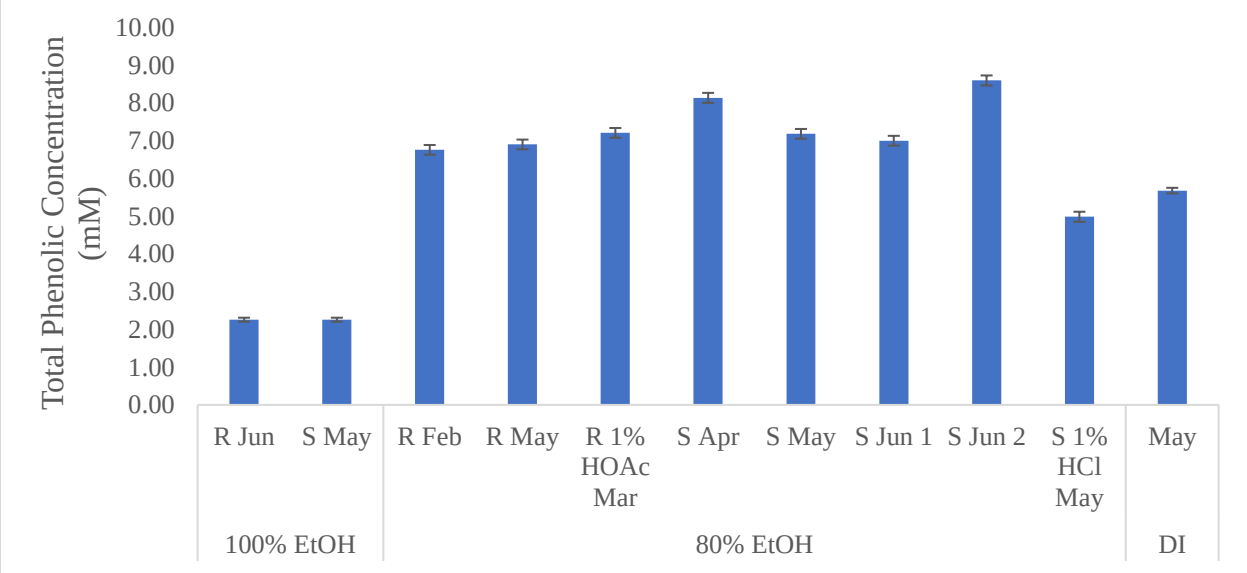

Figure 12: Total phenolic concentration (mM) in *Moringa oleifera* samples extracted in 100% ethanol, 80% ethanol, and DI water by refluxing (R) and shaking (S)

Capillary Electrophoresis

The compounds in *Moringa oleifera* leaves were separated using capillary electrophoresis with detection at 340 nm. There were many peaks present in the electropherograms the solutions with three main peaks with the largest peak being a broad peak. When the *Moringa oleifera* leaves were extracted in 80% ethanol by refluxing, the electropherogram had a higher absorbance than the electropherogram of the *Moringa oleifera* leaves extracted in 100% by refluxing (Figure 13). Similarly, the solution of *Moringa oleifera* leaves extracted in 80% ethanol by shaking had a higher absorbance than when extracted in water or 100% ethanol. The results suggest 80% ethanol is the most effective extraction solvent. The shaken solution extracted in 100% ethanol had much higher absorbance than the refluxed solution, which suggests that refluxing in 100% ethanol decreases the solubility of products or results in degradation or loss of products (Figure 14). Overall, extracting in 80% ethanol and refluxing resulted in the highest absorbance which suggests it is the most effective extraction method. The conditions need to be further optimized to achieve adequate separation of the peaks.

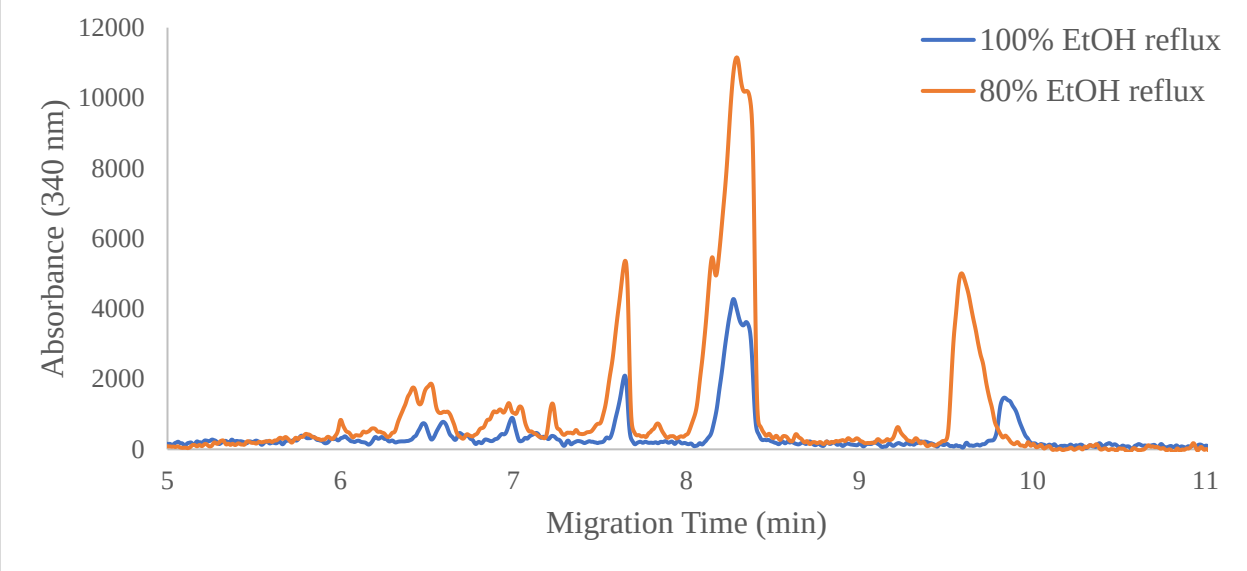

Figure 13: Electropherogram of *Moringa oleifera* extracted in 80% and 100% ethanol by refluxing injected at 0.5 psi for 10 seconds and separated at 20 kV with absorbance detection at 340 nm

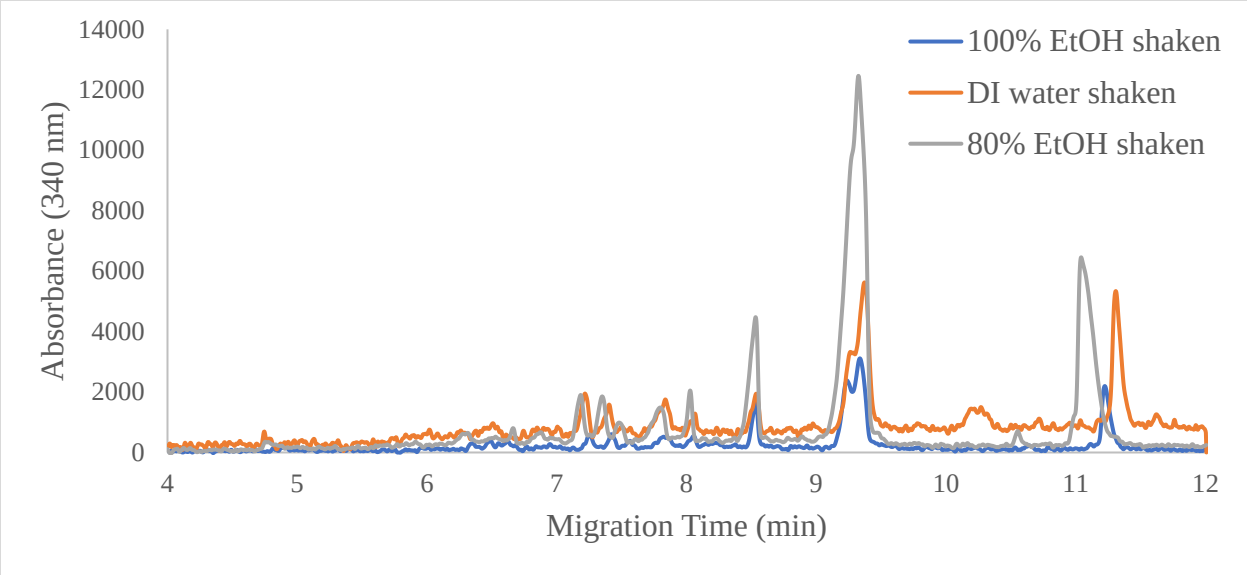

Figure 14: Electropherogram of *Moringa oleifera* extracted in 80% ethanol, 100% ethanol, and DI water by shaking injected at 0.5 psi for 10 seconds and separated at 20 kV with absorbance detection at 340 nm

Capillary Electrophoresis Optimization

Detection Wavelength

The detection wavelength can determine the peaks viewed on the electropherogram because it is dependent on the spectral properties of the compounds present in the sample. The electropherograms with detection at 254 nm and 340 nm had similar peak patters, but the larger peaks had a lower absorbance at 254 nm than 340 nm (Figure 15). Most aromatic compounds will absorb light at 254 nm, while flavonoid compounds also absorb at 340 nm. Using detection at 340 nm is preferred because it is more specific to flavonoids which are the compounds of interest. Acetone was used as the neutral marker (4.37 min) and is a sharp peak with detection at 254 nm but appears as a negative peak at 340 nm.

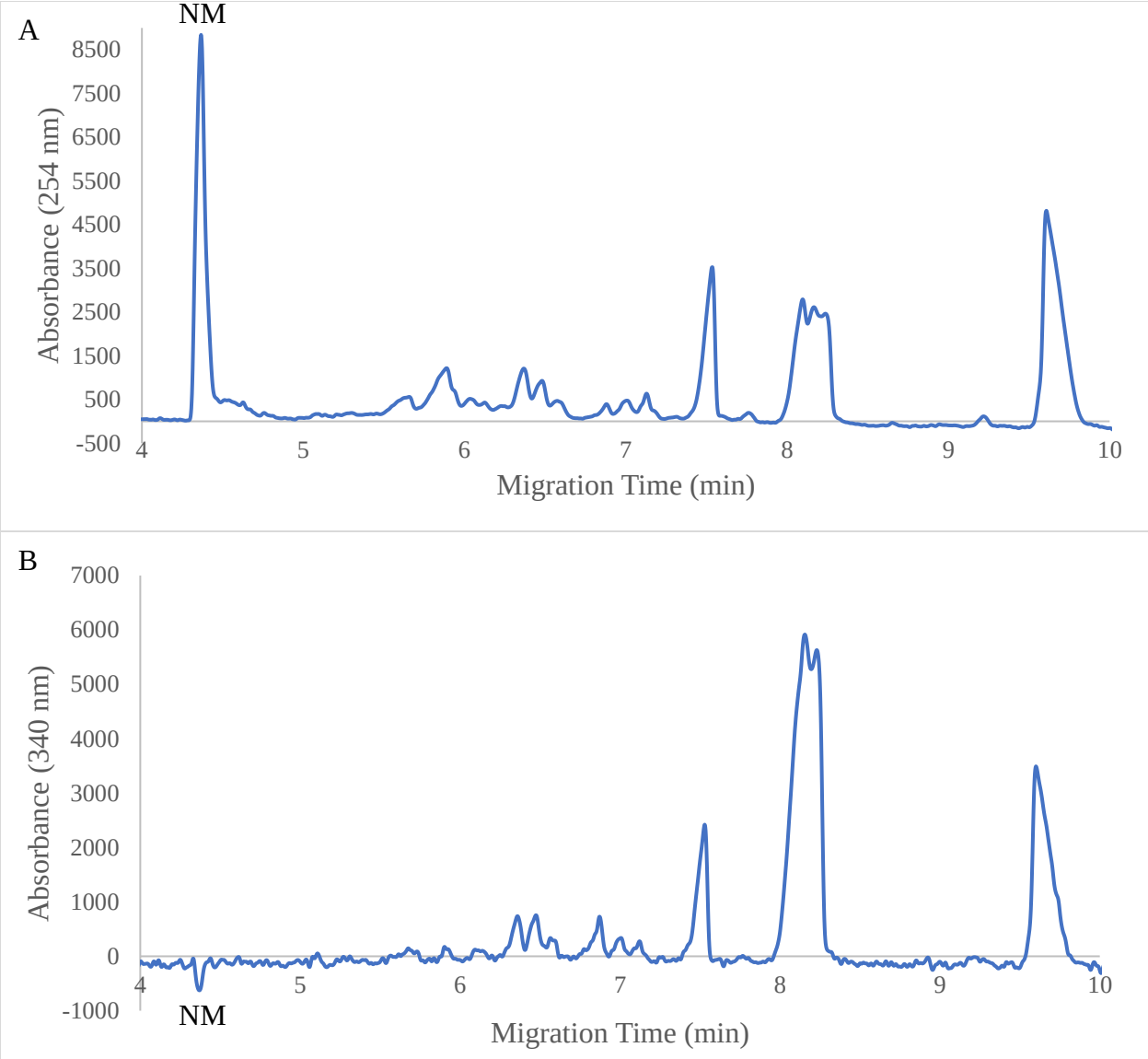

Figure 15: Electropherogram of *Moringa oleifera* leaves extracted in 80% ethanol by shaking injected at 1 psi for 5 sec and separated at 20 kV with absorbance detection at 254 nm (A) and 340 nm (B), NM – neutral marker, acetone

Separation Voltage

The voltage of applied to the sample to achieve separation affects the patterns of separation (Figure 16). Lower voltage results in greater separation between peaks. The change in separation voltage affects the largest peak while not greatly affecting the other peaks on the chromatograph. Separation at 20 kV was chosen as the most effective voltage because there is a

greater separation of the largest constituent peak as compared to separation at 30 kV, but there is not a significant difference in separation between separation at 20 kV and 15 kV except that separation at 15 kV took longer time.

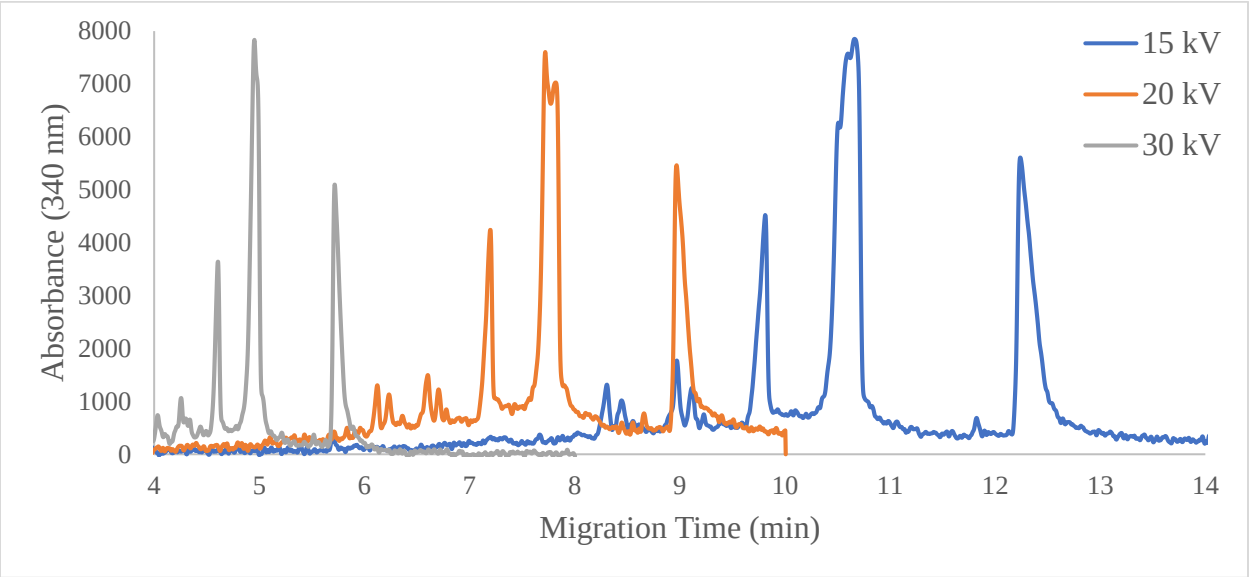

Figure 16: Electropherogram of *Moringa oleifera* extracted in 100% ethanol by shaking injected at 0.5 psi for 5 seconds and separated at 15 kV, 20 kV, and 30 kV with absorbance detection at 340 nm

Buffer pH

The pH of the buffer affects the separation of the compounds because the charge of the compounds, changes according to their pKa. The effect of the buffer pH on the separation of the compounds in *Moringa oleifera* was investigated (Figure 17). The sharpest peaks resulted in the electropherogram with the sample at pH 9.0. The sample at pH 9.5 also had sharp peaks, but the resolution between peaks better at pH 9.0. At pH 10.0 and 10.5, the peaks become less separated and defined. The optimal pH of the solvent is suggested to be 9.0, which other researchers have also concluded.[19]

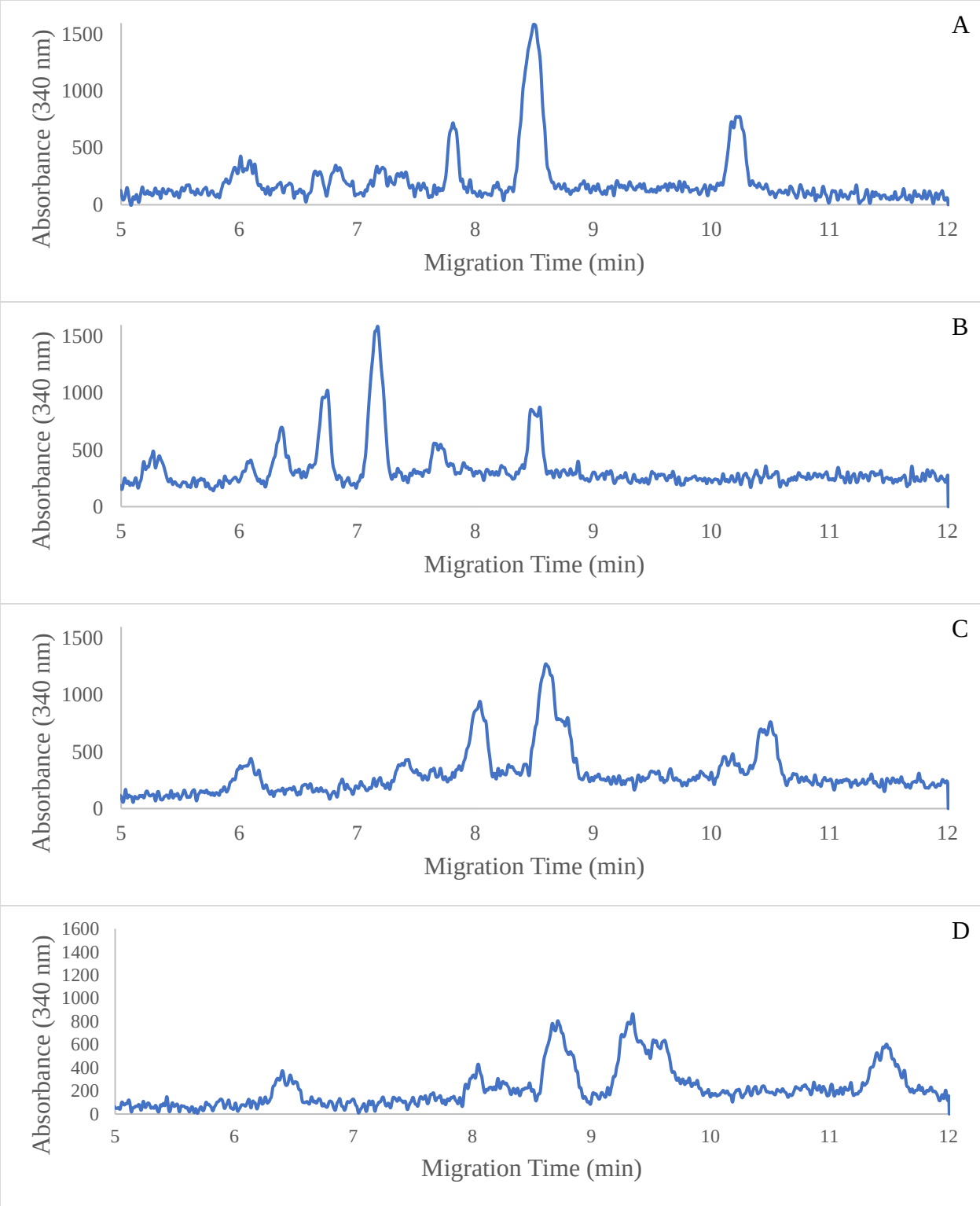

Figure 17: Electropherogram of *Moringa oleifera* extracted in 80% ethanol by shaking in 10 mM borate buffer pH 9.0 (A), pH 9.5 (B), pH 10.0 (C), and pH 10.5 (D) injected at 0.5 psi for 20 seconds and separated at 20 kV with absorbance detection at 340 nm

Neutral Marker

Acetone was investigated as neutral marker for detection at 340 nm (Figure 18). A sample of acetone in 10 mM borate buffer resulted in a negative peak at 5.16 min. When acetone was added to the *Moringa oleifera* sample, the negative peak was still present at 5.16 min. The peaks from the *Moringa oleifera* sample are detected after the neutral marker, which suggests that the compounds present in *Moringa oleifera* are negatively charged at pH 9.0.

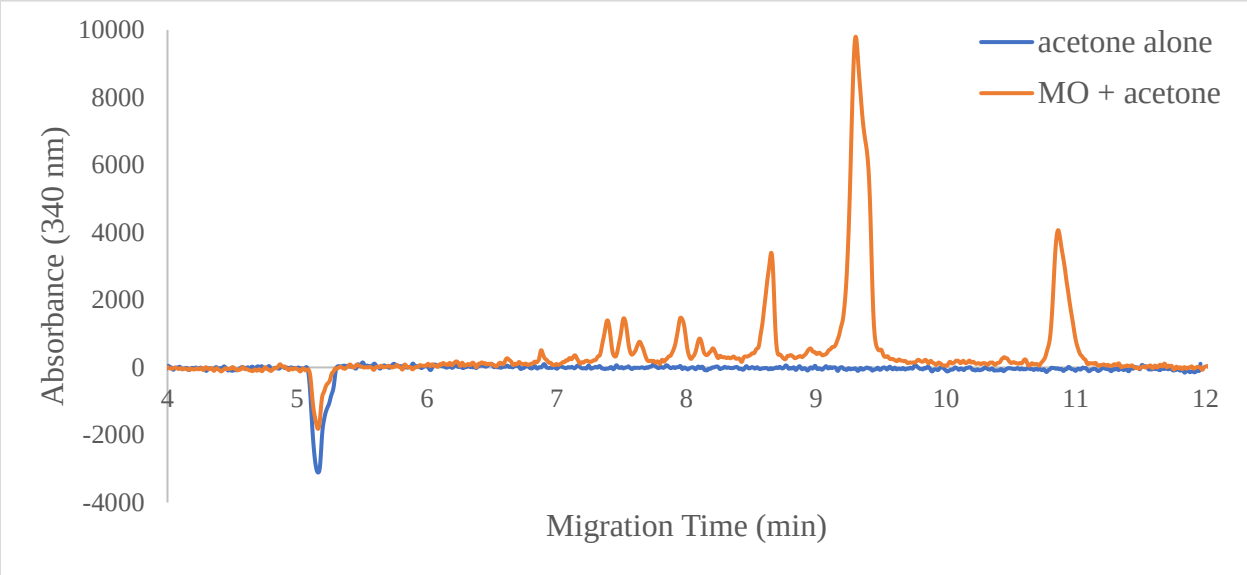

Figure 18: Electropherogram of acetone and *Moringa oleifera* spiked with acetone injected at 1 psi for 5 seconds and separated at 20 kV with absorbance detection at 340 nm

Capillary Electrophoresis Analysis

Reproducibility

The reproducibility of the *Moringa oleifera* extracts was determined by shaking two aliquots of *Moringa oleifera* powder in 80% ethanol (Figure 19). The electropherograms of the two samples resulted in a significant difference in the peak areas of three peaks (migration time [p-value]: 7.53 min [0.023], 8.16 min [0.044], and 9.60 min [0.027]), while there was no significant difference between the percent areas of the peaks in both electropherograms.

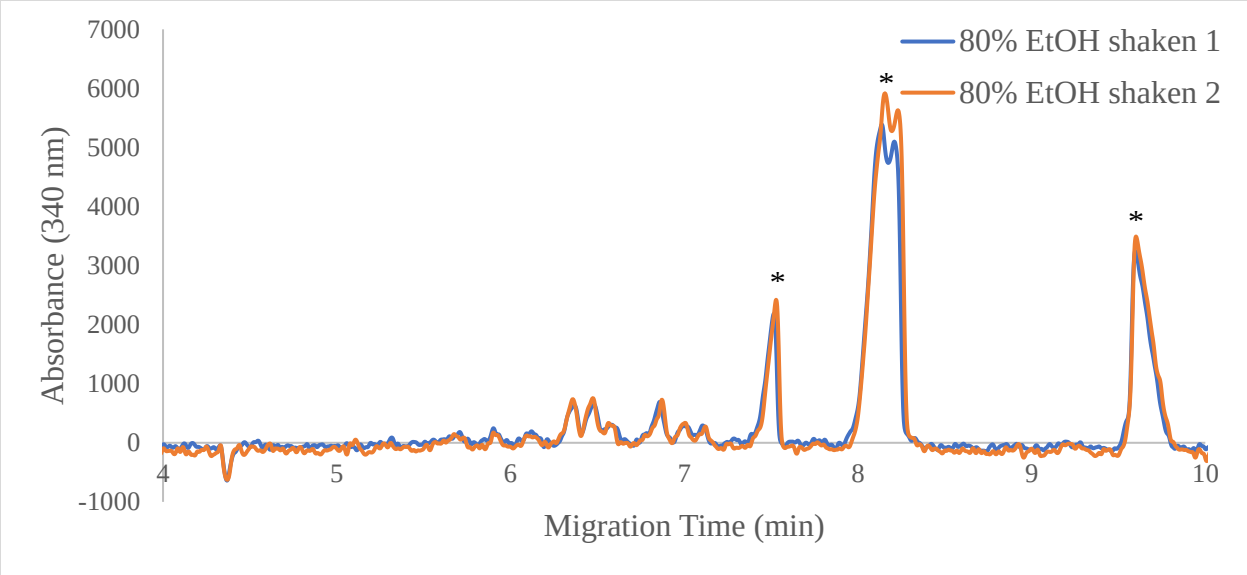

Figure 19: Electropherogram of independent *Moringa oleifera* extractions in 80% ethanol by shaking injected at 0.5 psi for 5 seconds and separated at 20 kV with absorbance detection at 340 nm (*peak areas p-value < 0.05)

Isoquercetin

A standard solution of isoquercetin in 10 mM borate buffer has a peak at 7.21 minutes (Figure 20). When isoquercetin is added to *Moringa oleifera*, the peak at 7.21 overlaps with a peak present in the electropherogram of *Moringa oleifera* without isoquercetin, suggesting the presence of isoquercetin in the *Moringa oleifera* sample.

A standard addition of isoquercetin to the *Moringa oleifera* leaf solution extracted in 80% ethanol shaken was completed for concentrations of isoquercetin from 0 µM to 750 µM (Figure 21). The resulting equation of the line was $y = [42 \pm 2]x + [9569 \pm 956]$ and R^2 of 0.986. The calculated concentration of isoquercetin in *Moringa oleifera* was 459 ± 78 µM after accounting for dilution. The results from this experiment need to be repeated to achieve greater accuracy in the measurement.

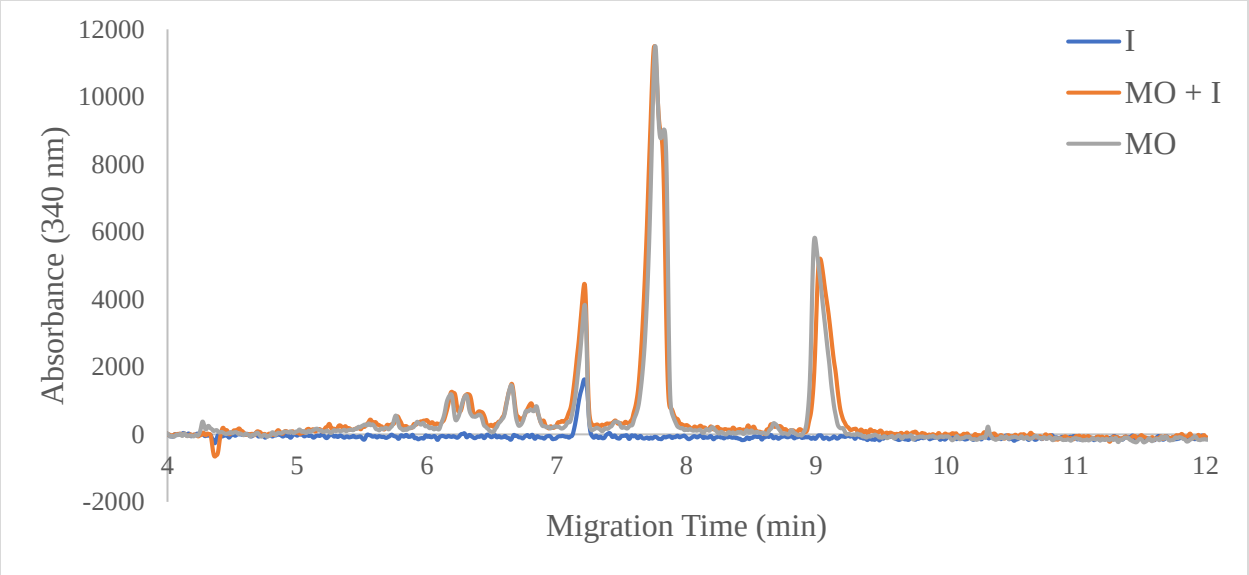

Figure 20: Electropherogram of isoquercetin (I), *Moringa oleifera* spiked with isoquercetin (MO + I), and *Moringa oleifera* (MO) injected at 1 psi for 5 seconds and separated at 20 kV with absorbance detection at 340 nm

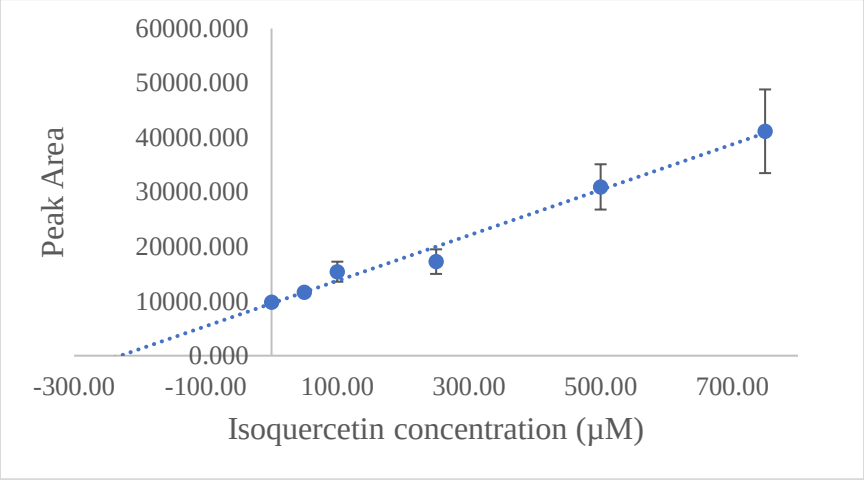

Figure 21: Standard addition calibration curve of isoquercetin peak area with detection at 340 nm with equation y = [42 ± 2]x + [9569 ± 956] and R^2 = 0.986

Rutin

A standard solution of rutin in 10 mM borate buffer appears as a peak at 6.75 minutes, but when rutin is added to *Moringa oleifera*, the peak overlaps with a peak present in the electropherogram of *Moringa oleifera* without rutin (Figure 22). The results suggest the presence

of rutin in *Moringa oleifera* leaves, but the concentration of rutin in *Moringa oleifera* still needs to be determined by standard addition.

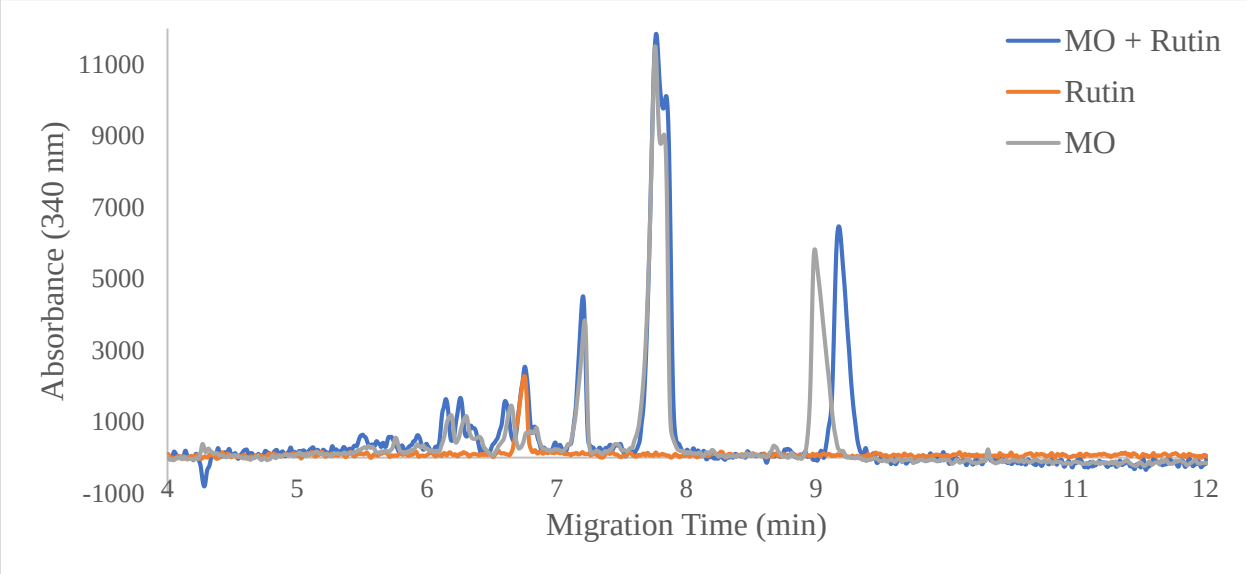

Figure 22: Electropherogram of rutin, *Moringa oleifera* spiked with rutin (MO + Rutin), and *Moringa oleifera* (MO) injected at 1 psi for 5 seconds and separated at 20 kV with absorbance detection at 340 nm

p-Coumaric acid

A standard solution of p-coumaric acid in 10 mM borate buffer has a peak at 8.75 minutes, but when *Moringa oleifera* is spiked with p-coumaric acid, the peak shifts to 8.66 min and does not overlap with a peak present in the electropherogram of *Moringa oleifera* without p-coumaric acid (Figure 24). The peak from p-coumaric acid overlaps slightly with a peak present on the *Moringa oleifera* electropherogram, which suggests that there is compound similar to p-coumaric acid present in *Moringa oleifera* leaves.

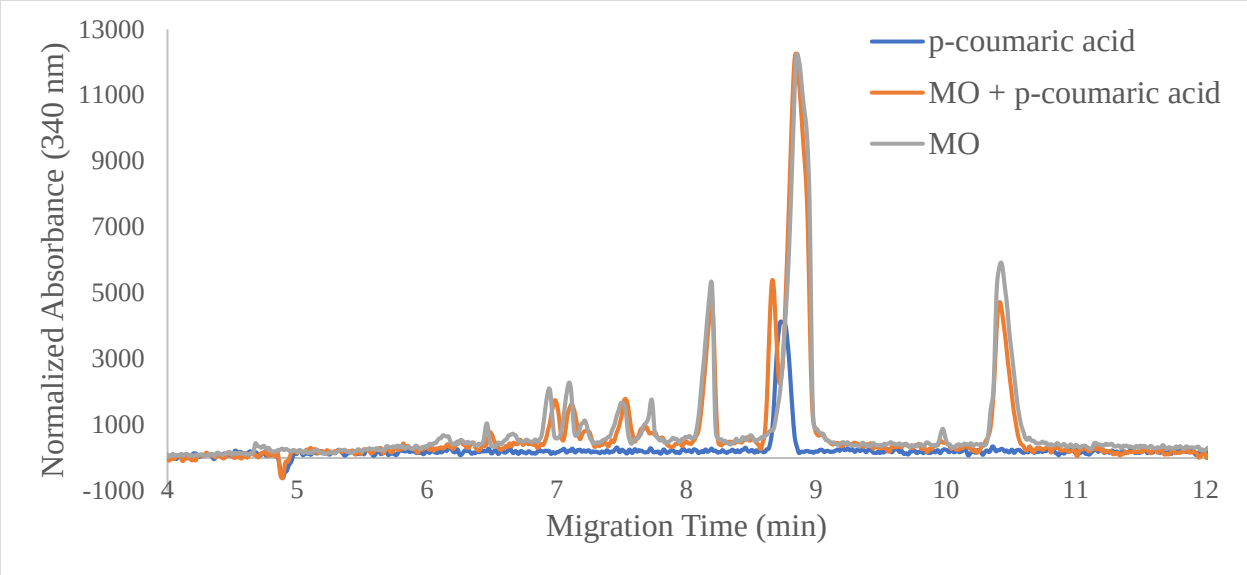

Figure 23: Electropherogram of p-coumaric acid, *Moringa oleifera* spiked with p-coumaric acid, and *Moringa oleifera* (MO) injected at 1 psi for 5 seconds and separated at 20 kV with absorbance detection at 340 nm

Conclusion

The photochemical content in *Moringa oleifera* leaves was analyzed with separation techniques using absorbance detection. Results from paper chromatography suggests the presence of photochemicals in *Moringa oleifera* leaves. The most effective extraction method was found to be shaking the *Moringa oleifera* leaves in 80% ethanol for 1 hour. The total phenolic content in *Moringa oleifera* leaves was measured to be 8.6 ± 0.1 mM. Capillary zone electrophoresis with detection at 340 nm was used to analyze the flavonoid content of *Moringa oleifera* leaves using acetone as the neutral marker. Rutin and isoquercetin were suggested to be present in the *Moringa oleifera* samples, while p-coumaric acid is most likely not present in *Moringa oleifera* leaves. The mean concentration of isoquercetin in *Moringa oleifera* leaves was calculated to be 459 ± 78 µM by standard addition.

Future Work

Future work will involve investigating the identity of other peaks within the electropherogram of *Moringa oleifera* leaves using standards and calculating the concentrations using a standard addition. Additionally, the identity of the compounds in *Moringa oleifera* leaves can be further confirmed using mass spectrometry. The total flavonoid content in *Moringa oleifera* leaves needs to be measures as well as a more accurate measurement for total phenolic content needs to be determined and optimized. The extraction and capillary zone electrophoresis procedures studied in this project could be used to evaluate the influence of environmental and growing conditions on the concentration of photochemicals in *Moringa oleifera* leaves. Eventually, the growing conditions could be standardized to produce a consistent concentration of flavonoids in the leaves of *Moringa oleifera* for large scale production.

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